Evaluating the effects of CUGBP1 deficiency in a mouse model of RNA toxicity

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Abstract

Myotonic dystrophy type 1(DM1), the most common form of adult-onset muscular dystrophy, is caused by an expanded $(CTG)_n$ repeat in the 3' untranslated region (UTR) of the DM protein kinase *(DMPK)* gene. The toxic RNA transcripts produced from the mutant allele alter the function of RNA binding proteins leading to the depletion of muscleblind-like (MBNL) proteins and an increase in steady state levels of CUG-binding protein 1 (CUGBP1). The role of increased CUGBP1 in DM1 pathogenesis is well studied using genetically engineered mouse models. However, the effect of reduction of CUGBP1 is not clear yet. In this study, we generated CUGBP1 knockout mice, which also carry an inducible toxic RNA transgene to test the effects of CUGBP1 reduction in RNA toxicity. We found that while absence of CUGBP1 did not correct splicing defects, it did however mitigate the increase in translational targets of CUGBP1 (MEF2A and CEBPβ). Notably, we found that loss of CUGBP1 prevented deterioration of muscle function by the toxic RNA, and resulted in better muscle histopathology. These data suggest reduction of CUGBP1 may be beneficial to the muscular dystrophy associated with RNA toxicity.

Introduction

Myotonic dystrophy type 1 (DM1) is the most common cause of the adult-onset muscular dystrophy with an incidence of 1 in 8000 and is a multi-systemic disorder (1). Major features of this autosomal dominant disorder are myotonia, muscle weakness, atrophy, smooth muscle dysfunction, cardiac defects and insulin resistance (2). DM1 is triggered by the expanded $(CTG)_n$ triple repeat in the 3'-untranslated region (UTR) of the *DMPK* gene (1). This is transcribed to produce mutant RNAs containing CUG repeats that are retained in the nucleus to form RNA foci (3, 4). This affects the nuclear and cytoplasmic activities of RNA binding proteins such as muscleblind-like 1 (MBNL1) and CUG-binding protein 1 (CUGBP1) (5-8).

MBNL1 binds to the expanded CUG repeat and co-localizes with the RNA foci, causing a local reduction of MBNL1 (6, 7). Consequently, the activity of MBNL1 as a splicing regulator is impaired, resulting in aberrant alternative splicing of target genes (9). Consistent with the loss of MBNL1 function, an analysis of MBNL1 knockout mice (*Mbnl*$^{\Delta E3/\Delta E3}$) showed that these mice developed some of the characteristic features of DM1, including misregulated mRNA splicing, muscle histopathological changes, cataracts, and myotonia (10). Moreover, an adenoviral delivery of MBNL1 reversed the splicing changes and myotonia, again underscoring the importance of MBNL1 in the DM1 disease phenotype (11).

In addition to MBNL1, CUGBP1, a member of CUG-BP and ETR-3-like factor (CELF) family, is implicated in the disease process. CUGBP1 has been reported to have multiple functions in RNA metabolism including regulation of alternative splicing, RNA stability and translational regulation of its RNA targets (12-16). The mutant *DMPK* transcript is thought to activate PKC activity, leading to a hyperphosphorylation of CUGBP1 (17), resulting in the stabilization of and increased steady state levels of CUGBP1 in DM1 skeletal muscle and heart tissues. Transgenic mice over-expressing CUGBP1 showed neonatal lethality, skeletal muscle histological changes, and misregulated splicing pattern observed in DM1 patients (18, 19). Heart specific over-expression of CUGBP1 caused premature lethality, histopathological and echocardiographic abnormalities, and splicing defects (20). Two different transgenic mouse models which over-expressed CUGBP1 in skeletal muscle developed DM1 features. One mouse model showed that CUGBP1 responsive alternative splicing events were misregulated in skeletal muscle and that CUGBP1 affects muscle integrity and function (21). In the other model, muscular dystrophy, fiber type switching, and delayed muscle development were seen in conjunction with increased levels of p21 and MEF2A (both of which are translational targets of CUGBP1) (18). Both of these models exhibited muscle loss, impaired muscle function and dystrophic muscle histology. These data demonstrate that increased CUGBP1 contributes to DM1 pathogenesis and suggests that reduction of CUGBP1 in a DM1 mouse model may have beneficial effects.

To test this, we generated CUGBP1 knockout mice that also carried an inducible toxic RNA transgene (5-313) (22). We found muscle function in these double transgenic mice was protected and the histopathological features were milder as compared to mice carrying only the toxic RNA transgene, despite the fact that many alternative splicing events known to be mis-regulated in DM1 were not corrected by the absence of CUGBP1. This was associated with decreased *Nkx2-5* levels in skeletal muscle from *Cugbp1*$^{-/-}$ mice expressing the toxic RNA, and corresponded to the milder histopathology of the muscle in these mice. Additionally, the protein levels of MEF2A and C/EBPβ were reduced in the absence of CUGBP1. These data suggest reduction of CUGBP1 may be beneficial to the muscular dystrophy associated with DM1.

Results

Levels of CUGBP1 correlate with skeletal muscle histopathology in DM1.

We first evaluated CUGBP1 levels in skeletal muscles from patients with DM1. Western blot analyses of muscle extracts showed increased CUGBP1 (up to 5 fold) that correlated well with muscle histopathology (Fig. 1A). Next, we investigated CUGBP1 in a doxycycline inducible RNA toxicity mouse model of DM1 (5-313), which expresses an eGFP gene fused to the *DMPK* 3'UTR $(CTG)_5$ (22). CUGBP1 was increased by induction of toxic RNA (between 1.5 to 5 fold) and the expression was highest in the muscles with the most severe histopathology (Fig. 1B). Thus the mouse model accurately represented these aspects of the disease phenotype providing a basis for further investigations.

Absence of CUGBP1 does not affect MBNL1 or CUGBP2 in mice with RNA toxicity.

To determine whether reduction of CUGBP1 will improve the muscle pathology, 5-313 mice were crossed to *Cugbp1*$^{-/-}$ mice to generate *Cugbp1*$^{-/-}$/5-313$^{+/-}$ mice and *Cugbp1*$^{-/-}$/5-313$^{+/+}$ mice. The ratio of

+/+, +/- and -/- *Cugbp1* mice was different from the expected 1:2:1 Mendelian ratio at 21 days after birth (+/+: +/-: -/- = 24: 62: 14, $P < 0.0001$, χ^2 test). This result is very similar to a previous *Cugbp1*$^{-/-}$ mice study (23). *Cugbp1*$^{-/-}$ mice were weak and developed cataracts (Supp. Fig. S1). Both *Cugbp1*$^{-/-}$/5-313$^{+/-}$ and *Cugbp1*$^{-/-}$/5-313$^{+/+}$ were induced to express the toxic RNA using 0.2% doxycycline delivered in the drinking water for 5 months and 2 weeks, respectively. *Cugbp1*$^{+/+}$/5-313 induced (+dox) and the appropriate uninduced (-dox) controls were also utilized. First, we analyzed the expression of *Cugbp1* by qRT-PCR (Supp. Fig. S2A) and western blot (Fig. 2) to ensure knockout mice did not express any CUGBP1. *Cugbp1*$^{+/+}$/5-313 control mice expressed 2-3 fold more CUGBP1 after the induction of toxic RNA, whereas CUGBP1 was not detected in *Cugbp1*$^{-/-}$/5-313 mice even after induction (Fig. 2). Induction of the transgene was confirmed by qRT-PCR (Supp. Fig. S2B) and western blot for eGFP (Fig. 2).

We tested whether any of the other CELF proteins compensated for the absence of CUGBP1. The level of *Cugbp2* expression was determined using qRT-PCR (Supp. Fig. S2C) and western blot (Fig. 2). Both *Cugbp2* mRNA and protein levels of CUGBP2 were unaffected by the absence of CUGBP1 and the presence of toxic RNA. We also performed qRT-PCR to quantify the levels of *Celf4* expression and found it was not expressed in skeletal muscle (data not shown). MBNL1 protein levels were assayed by western blot. Neither RNA toxicity nor the absence of CUGBP1 changed the levels of total MBNL1 protein in this mouse model (Fig. 2).

Absence of CUGBP1 preserves muscle function in the presence of RNA toxicity

To analyze muscle function, we have established treadmill running and grip strength tests. *Cugbp1*$^{-/-}$/5-313 mice were weak even in the absence of toxic RNA. Furthermore, induction of RNA toxicity by doxycycline administration in 5-313$^{+/+}$ mice made them much weaker leading to difficulties measuring the muscle function such as running and grip strength. Due to the severity of this animal model, *Cugbp1*$^{-/-}$

/5-313$^{+/-}$ mice, which developed a milder disease phenotype, were used instead and tested for grip strength and their ability to complete a 752 meter forced run. After 5 months, the *Cugbp1*$^{+/+}$/5-313$^{+/-}$ uninduced (-dox) group retained 99% of their run ability and 88.2% of their forelimb grip strength compared to their baseline function, whereas *Cugbp1*$^{+/+}$/5-313$^{+/-}$ induced for 5 months with continued doxycycline administration (+dox) mice decreased to 83.6% and 75.9%, respectively (Fig. 3A, B) ($p < 0.05$). *Cugbp1*$^{-/-}$/5-313$^{+/-}$ uninduced (-dox) mice retained only 31.3% of their run ability and 77.5 % of their grip strength compared to their baseline function. However, the *Cugbp1*$^{-/-}$ mice expressing the toxic RNA maintained their run ability (29.9%) and their grip strength (82.1%) (Fig. 3A, B). Thus unlike the RNA toxicity mice expressing CUGBP1 where we observed a significant decrease in function, the loss of CUGBP1 resulted in a preservation of muscle function in the 5-313 $^{+/-}$ transgenic mice with RNA toxicity. We also examined cardiac function by ECG and myotonia using EMG. Both *Cugbp1*$^{+/+}$ and *Cugbp1*$^{-/-}$ mice developed prolonged PR intervals on the ECG and myotonia by two weeks after induction. (Supp. Table S1). Absence of CUGBP1 did not improve the cardiac conduction defects or myotonia, in the induced *Cugbp1*$^{-/-}$ /5-313 $^{+/-}$ mice.

Absence of CUGBP1 does not correct misregulated alternative splicing.

Misregulation of developmental alternative splicing transitions is a characteristic molecular feature of DM1, and CUGBP1 is known to play an important role in alternative splicing (6, 19, 24-26). Some of the splicing targets are antagonistically regulated by MBNL1 and CUGBP1 while others are specific to MBNL1 or CUGBP1 (13, 27). To determine whether the removal of CUGBP1 restores the missplicing events affected by the toxic RNA, we analyzed various splicing targets which are abnormally spliced in the 5-313$^{+/+}$ model. Aberrant splicing of *Clcn-1* exon 7 is a key feature of DM1 and responsible for myotonia (28, 29). Both *Cubgp1*$^{+/+}$ and $^{-/-}$ mice showed misregulation of *Clcn-1* splicing in the presence of toxic RNA (Fig. 4A). Additionally *Tnnt3* exon F (the fetal isoform) and *Smyd1* exon 39 were

misregulated in skeletal muscle from both induced +/+ and -/- mice (24, 30, 31) (Fig. 4A). Splicing targets reported to be CUGBP1 specific (*Nrap* exon 12 and *Fxr1h* exon 15, 16) were also examined and found to be misregulated by toxic RNA in both the +/+ and -/- mice (13, 21, 32-34) (Fig. 4B). In summary, the alternative splicing events that are misregulated in this RNA toxicity model were not rescued by knockout of CUGBP1.

Interestingly, we found that the absence of CUGBP1 lead to a more embryonic pattern in all splicing events compared with mice expressing CUGBP1, even in the absence of toxic RNA (Fig. 4A, B). To determine if the muscle from *Cugbp1*$^{-/-}$ mice is in an earlier embryonic/developmental stage, we looked at the expression of embryonic myosin heavy chain (*Myh3*) by qRT-PCR. *Cugbp1*$^{-/-}$ mice have 3.6 fold ($P < 0.05$) more *Myh3* than *Cugbp1*$^{+/+}$ mice in the absence of RNA toxicity and in the absence of any active degeneration/regeneration process (Fig. 4C). This is consistent with a more developmentally immature muscle. The levels increased to 6.7 fold after induction of RNA toxicity (Fig. 4C), like the responses seen in mice with CUGBP1. These data suggest that CUGBP1 is not as strong a splicing modulator in RNA toxicity, and that other factors affected by toxic RNA (e.g. MBNL1) may have a greater impact on the splicing events.

Absence of CUGBP1 leads to better muscle histology.

Hematoxylin and eosin staining were performed on quadriceps muscles from *Cugbp1*$^{+/+}$/5-313 and *Cugbp1*$^{-/-}$/5-313 mice. These muscles were graded according to a histopathology scoring system we have developed in the lab. Both uninduced *Cugbp1*$^{+/+}$ and $^{-/-}$ mice showed normal muscle histology with peripherally located nuclei and uniform fiber size (Fig. 5A). Atrophic fibers and inflammation were not observed in these muscles. By 2 weeks on 0.2% dox induction, *Cugbp1*$^{+/+}$/5-313$^{+/+}$ mice had characteristic DM1 histological feature including increased central nuclei, variation in fiber size and nuclear clumping (Fig. 5A), as described previously (22). Their resulting average scores fell within the

moderate severity range (Fig. 5B). However, the histology scores of induced *Cugbp1*$^{-/-}$ mice fell within the mild range. In these muscles, only 2-3% of the fibers contained central nuclei and these mice had minimal fiber size variation and no nuclear clumping (Fig. 5A).Thus the severity of *Cugbp1*$^{-/-}$/5-313$^{+/+}$ induced (+dox) mice was milder than the *Cugbp1*$^{+/+}$/5-313$^{+/+}$ (+dox), ($P < 0.02$, Mann-Whitney U-test). Although removal of CUGBP1 did not lead to completely normal muscle histology, it did result in significantly improved muscle histopathology.

We have observed that *Nkx2-5* level correlate with severity of the muscle histopathology in DM1 (data not shown). We examined *Nkx2-5* levels in *Cugbp1*$^{+/+}$/5-313 and *Cugbp1*$^{-/-}$/5-313 mice using qRT-PCR. *Nkx2-5* mRNA levels in the induced *Cugbp1*$^{+/+}$/5-313 mouse muscles were dramatically increased, whereas the levels were undetectable in the absence of RNA toxicity (Fig. 5C). However, the induced *Cugbp1*$^{-/-}$ mice had 5 times less *Nkx2-5* expression relative to the induced *Cugbp1*$^{+/+}$ mice (Fig. 5C). Downstream targets of *Nkx2-5 like Gata4*, *Nppa* and *Nppb* (31), were assessed by qRT-PCR and their levels were likewise affected (Supp. Fig. S3).

Translational targets of CUGBP1 are also affected in skeletal muscle

CUGBP1 functions as a translational regulator for several genes such as MEF2A and the C/EBPβ. It has been reported that transgenic mice over-expressing CUGBP1 have elevated levels of MEF2A and a delay in myogenesis (18). To examine whether the level of these translational targets are affected by the presence of RNA toxicity and CUGBP1 status, we analyzed the expression of MEF2A and C/EBPβ by western blotting. We found that CUGBP1 status had no significant effect on the levels of MEF2A in uninduced mice. However, the level of MEF2A increased in the presence of RNA toxicity in mice wildtype for *Cugbp1* and this effect was absent in the muscles of *Cugbp1*$^{-/-}$/5-313$^{+/+}$ (+dox) mice (Fig. 6A). Likewise, both of the isoforms of C/EBPβ (LAP and LIP) were consistently increased in *Cugbp1*$^{+/+}$/5-313$^{+/+}$ (+dox) mice with RNA toxicity in several independent experiments (Fig. 6B, C).

Notably, the levels of both the LAP and LIP isoforms were not significantly induced in *Cugbp1*$^{-/-}$/5-313$^{+/+}$ (dox+) mice as compared to uninduced mice, and were significantly less than 5-313$^{+/+}$ (dox+) mice that expressed CUGBP1 (Fig. 6B, C).

It has been previously reported that glucocortcoid treatment of mice increased C/EBPβ in skeletal muscle and regulated factors of muscle atrophy, and that knockdown of C/EBPβ reduced these levels in myoblasts (36). Given the improved muscle histology and decreased levels of C/EBPβ in the *Cugbp1*$^{-/-}$/5-313 with RNA toxicity, we next analyzed *atrogin-1* and *MuRF1* mRNA levels in our mouse model. Neither toxic RNA nor absence of CUGBP1 influenced *atrogin-1* mRNA in our mouse model (data not shown). However, RNA toxicity increased *MuRF1* expression and this was alleviated in *Cugbp1*$^{-/-}$/5-313$^{+/+}$ mice (Fig. 6D). Thus, absence of CUGBP1 in the toxic RNA model mitigated the effect on translational targets like MEF2A and C/EBPβ, which are thought to play a role in myogenesis and muscle atrophy.

Discussion

Several studies using CUGBP1 transgenic mice support a role for CUGBP1 in DM1 pathogenesis (18-21, 37). Transgenic mice over-expressing CUGBP1 reproduced splicing defects observed in individuals with DM1 and cardiac or muscle specific expression also showed histological and functional DM1 features (19-21). Recently, a study using a dominant negative CUGBP1 protein showed rescue of splicing defects in a cell culture system and a DM1 mouse model (34). In addition, transgenic mice over-expressing a His-tagged CUGBP1 had increased mortality and evidence of disrupted myogenesis, a feature found in congenital DM1 (18).

These studies also supported the notion that the levels of CUGBP1 have a strong correlation with disease severity (18, 21). Mice with 2-3 fold elevation of His-CUGBP1showed mild histological changes such as increased numbers of central nuclei, whereas higher levels (4-6 fold) leads to fiber type switching, fiber size variation as well (18). In another study, using and inducible CUGBP1 transgenic mouse model, an 8-fold increase in CUGBP1 led to severe histopathology in 4 weeks, whereas a lower induction of CUGBP1 (2-fold) resulted in very mild muscle pathology (21). In our mouse model as well as patient tissues, we also see a clear correlation between CUGBP1 levels and muscle pathology (Fig. 1). This suggests that reduced levels of CUGBP1 could result in better muscle histology.

As a prelude to performing experiments presented here, we had done a similar series of experiments using *Cugbp1*$^{+/-}$ (i.e. heterozygous knockout mice) and found no significant effects on the measured phenotypes or on CUGBP1 levels (data not shown). So, we examined the consequences of CUGBP1 deficiency in RNA toxicity by producing a complete knockout of CUGBP1 in our inducible RNA toxicity model. The absence of CUGBP1 by itself (i.e. *Cugbp1*$^{-/-}$ mice) resulted in weak and small mice that had deficits in running on a treadmill and in grip strength assays (Fig. 3). Also, these mice developed cataracts, a phenotype commonly seen in patients with DM1 (Supp Fig. 1). This is unlikely to be related to the pathogenesis of cataracts in DM1 since CUGBP1 levels are elevated in DM1, not diminished or absent. Notably, absence of CUGBP1 did not lead to myotonia, obvious cardiac conduction defects, or obvious histopathology in skeletal muscles; phenotypes that are all prominent in DM1. However, there was some evidence for muscle immaturity based on RNA splicing patterns that resembled a more embryonic developmental stage and increased expression of embryonic myosin heavy chain isoforms (Fig. 4).

Having established a baseline phenotype for the CUGBP1 deficient mice, our goal was to see what effect CUGBP1 deficiency would have on our RNA toxicity model. CUGBP1 has been reported to have multiple functions in RNA metabolism including regulation of alternative splicing, RNA stability and

translational regulation of its RNA targets and a number of molecular events have been associated with increased CUGBP1 and RNA toxicity. Using a systematic analysis of these associated phenotypes, we found that absence of CUGBP1 had no obvious effect on the myotonia or cardiac conduction defects caused by RNA toxicity. Surprisingly, disrupted alternative splicing, a key feature of DM1 pathogenesis, is not corrected by reduction of CUGBP1. Not only general targets like *Clcn-1* and *Tnnt3*, but also reported CUGBP1 specific targets (*Nrap* and *Fxr1h*) are mis-regulated by the toxic RNA even in the absence of CUGBP1. Though CUGBP1 and MBNL1 have been thought of as mutual antagonists, one possibility is that MBNL1 may be the stronger and more dominant modulator of alternative splicing defects in the RNA toxicity associated with DM1. Although RNA foci and sequestration of MBNL1 are not observed in 5-313 mouse muscle, it is possible that submicroscopic foci exist due to an interaction with MBNL1. Using RNA IP of MBNL1 and associated RNAs, we have found that MBNL1does indeed interact with the *GFP-DMPK* 3'UTR $(CUG)_5$ RNA in our mice and both the normal and mutant *DMPK* mRNAs in human DM1 cell lines (data not shown). Although we did not see any compensatory effect of CUGBP2 or other CELF proteins, it is also possible that the toxic RNA could affect other unknown factors that may play a role in RNA splicing in this mouse model.

Though the CUGBP1 deficient mice started off weaker, the fact that there was no further diminution in the muscle function tests (treadmill run and grip strength) when RNA toxicity was induced in these mice, unlike the mice that were expressing CUGBP1 (Fig. 3), showed that CUGBP1 deficiency preserved muscle function despite the effects of RNA toxicity. Additionally, abnormal muscle histology in mice expressing the toxic RNA was restored towards normal, resulting in mice with milder histopathology (Fig. 5). Thus absence of CUGBP1 seems to have a clear protective effect against these adverse effects of RNA toxicity. As well, a number of molecular changes caused by RNA toxicity were minimized by the absence of CUGBP1. For instance, it has been previously proposed that the cytoplasmic function of CUGBP1 as a translational regulator could be disrupted by RNA toxicity (18). In this model, it is posited

that CUGBP1 post-transcriptionally regulated various target mRNAs, such that in DM1, increased CUGBP1 in muscle tissue disrupted myogenesis via up-regulation of translational targets such as MEF2A (18) and C/EBPβ (38). Indeed, in our RNA toxicity model, we see significant elevation of both MEF2A and C/EBPβ in skeletal muscle along with an increase in CUGBP1. Of note, we find that in the absence of CUGBP1 in our RNA toxicity mouse model, there is no significant increase in MEF2A and C/EBPβ and that this correlates with a beneficial effect on the histopathology and muscle function.

Though it is unclear how the effects on these translational targets could be affecting muscular dystrophy in our model, several recent studies have implicated C/EBPβ in myogenesis and muscle wasting. In a recent study it was found that dexamethasone administration to cultured myoblasts led to increased C/EBPβ, upregulated *atrogin-1* and *MuRF1* and reduced cell size (36). Treating the myoblasts with a siRNA against C/EBPβ resulted in reductions in C/EBPβ and MuRF1and was associated with preservation of cell size. RNA toxicity in our mouse model increases C/EBPβ and *MuRF1* levels in skeletal muscle and is associated with muscle weakness, atrophy and histopathology. Of note, the absence of CUGBP1 reduced C/EBPβ and *MuRF1* and was associated with decreased atrophy and histopathology in our mouse model, analogous to the results from the myoblast study. Furthermore, another group recently reported that C/EBPβ was expressed in satellite cells in skeletal muscle and that increased expression of C/EBPβ inhibited satellite cell and C2C12 myoblast differentiation and that loss of C/EBPβ in satellite cells promoted muscle differentiation (39). This may have relevance to DM1 muscle histopathology as it has been reported that satellite cell numbers were increased in muscles from DM1 patients without a concomitant increase in muscle regeneration, suggestive of a block in satellite cell activation or subsequent recruitment into a differentiation program (40). This is also consistent with reports of defects in differentiation and maturation in satellite cells from patients with DM1 (40, 41).

In summary, CUGBP1 up-regulation clearly correlates with severity of muscle pathology in DM1 patients and in our mouse model of RNA toxicity. However, our data suggests that even complete removal of CUGBP1 does not have a significant effect on key phenotypes of DM1 including myotonia, cardiac conduction defects and surprisingly, even several splicing defects. Thus, therapeutic approaches that target only CUGBP1 seem likely to have serious limitations, as *Cugbp1*$^{-/-}$ mice are small and weak and key DM1 phenotypes persist even in the absence of CUGBP1. However, reductions in CUGBP1 did have a beneficial effect on muscle histopathology and preservation of muscle function perhaps through alterations in the functions of CUGBP1 as a translational regulator. This suggests that targeting CUGBP1 could have limited therapeutic benefits with respect to the muscular dystrophy in DM1. But it is likely that this would have to be undertaken in a combinatorial approach with compounds that target MBNL1 sequestration, or the toxic RNA, or other novel pathways affected by RNA toxicity in patients with DM1.

Materials and methods

Mouse models.

All animals were used in accordance with protocols approved by the Animal Care and Use Committee (ACUC) at the University of Virginia. DM1 mouse models were generated as previously described (22). All progenies were genotyped by PCR. Six weeks old mice were induced with 0.2 % of doxycycline in drinking water. 5-313$^{+/-}$ mice were used for the running and the grip strength test. The remaining experiments used 5-313 $^{+/+}$ mice.

Protein isolation and western blot.

Tissues were homogenized in RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP-40, 0.5% Na –deoxycholate, 0.1% SDS) containing protease inhibitor and phosphatase inhibitor. Protein concentration

was determined by Bradford assay with 20-40 μg of total protein per lane used for western blotting. Proteins were detected with antibodes against the following antibodies: CUGBP1 (3B1, EMD Millipore), CUGBP2 (N-15, Santa Cruz Biotechnology (SCBT)), MBNL1 (A2764, gift of C.A. Thornton), MEF2 (C21, SCBT) and C/EBPβ (C-19, SCBT).

Treadmill assay and grip strength test.

Mice were examined for running endurance on a treadmill (Columbus Instruments, OH). Mice were started for 1 min at 10m/min and then ran at the speed of 15m/min for 2 min. The treadmill accelerated to 29 m/min at a rate of 2m/min/2min. After 17min, the treadmill was then increased to 30 m/min for 13min. When the mice reached exhaustion (defined as greater than 5 consecutive seconds on the shock grid without attempting to re-engage the treadmill), they were removed from the device. Total time and total distance were recorded for each trial (Supp. Table S2).

Grip strength was measured using a digital grip strength meter, which records the maximal strength an animal exerts while trying to resist an opposing pulling force (Columbus Instruments, Columbus, OH). Forelimb grip strength was measured using a mouse tension bar. The results of five consecutive trials on the same day were averaged for each animal. Testers were blind to genotype.

ECGs and EMGs.

ECG and EMG were measured as described previously (35). Mice were anesthetized with intraperitonial valium (2.5 mg/kg body weight) and ketamine (100 mg/kg body weight) and kept warm during the entire procedure. EMGs were carried out with a TD-20 MK1 EMG machine from TECA, using subdermal electrodes for stimulation, and grounding. Three lead ECG was performed with a BioAmp/Powerlab from ADInstrument. All protocols were approved by and performed under the auspices of the UVA Institutional Animal Care and Use Committee.

RNA isolation and RT-PCR.

Total RNA was extracted from skeletal muscle tissues using TRIzol reagent (Invitrogen). cDNA was synthesized from 1μg RNA using QuantiTech Reverse Transcription Kit (Qiagen) and then subjected to PCR using gene-specific primers. After separation on a 1% agarose gel or 10% acrylamide gel, bands were quantified using Kodak gel imaging and ImageQuant.

Quantitative RT-PCR (qRT-PCR) was performed using the BioRad iCycler and detected with SYBERGreen dye. Primer sequence and PCR conditions are given in Supp. Table S3. All assays were done in duplicate, and data normalization was accomplished using an endogenous control (Gapdh). The values were subjected to a $2^{(-\Delta\Delta Ct)}$ formula to calculate the fold change between the control and experimental groups.

Histology.

Quadriceps femoris muscles were collected in isopentane and frozen in liquid nitrogen. Tissues were cut at 6μm with a cryostat. Hematoxyline and eosin staining was done according to standard procedures and examined under a light microscope.

Statistics

All data are expressed as mean ± standard deviation. Statistical significance was determined using a two-tailed Student's *t*-test with equal or unequal variance as appropriate. Mann-Whitney U test was used for non-parametric samples and χ^2 test was used for multinomial samples. All statistical analyses were performed with Microsoft Excel and Minitap16 software. The significant level was set at *P*-values less than 0.05 for all statistical analyses.

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Figure Legends

Figure 1. CUGBP1 levels correlate with muscle histopathology in skeletal muscles of patients with DM1 and in the 5-313 mice. Graded muscle tissues from **(A)** human samples and **(B)** the 5-313 mouse model were subjected to western blot for CUGBP1. CUGBP1 levels increased with severity of muscle histopathology in both DM1 individuals and the 5-313 mice. GAPDH was used as loading control.

Figure 2. Molecular characterization of RNA-binding proteins. Protein extracts were prepared from skeletal muscle tissues of mice and examined by western blots. CUGBP1 was not detected in *Cugbp1*$^{-/-}$ mice. Western blot for CUGBP2, MBNL1 showed no change due to absence of CUGBP1 or presence of RNA toxicity. Western blot for eGFP confirmed transgene induction. GAPDH was used as loading control.

Figure 3. Preservation of muscle function in *Cugbp1* $^{-/-}$/5-313$^{+/-}$ mice. *Cugbp1*$^{+/+}$/5-313$^{+/-}$ mice (n=7) and *Cugbp1*$^{-/-}$/5-313$^{+/-}$ mice (n=5) were induced with 0.2% of doxycycline for 5 months. Uninduced mice were used as control (*Cugbp1*$^{+/+}$/5-313$^{+/-}$ (n=4), *Cugbp1*$^{-/-}$/5-313$^{+/-}$ (n=3)). **(A)** Mice were subjected to

treadmill running and distance run was measured. The measurements were converted to % retained, as compared to a baseline measurement for each mouse. **(B)** Grip strength was assessed using a grip strength meter. Data were expressed as force in grams and converted to % of grip-strength retained as compared to baseline. Significant differences were $**P < 0.01$ and $***P < 0.005$ (t-test).

Figure 4. Absence of CUGBP1 has no beneficial effects on the RNA splicing defects caused by RNA toxicity. (A) *ClCn-1* exon 7a, *Tnnt3*-fetal exon and *Smyd1* exon 39 were analyzed as mRNA splicing targets of DM1. **(B)** *Nrap* exon 12 and *Fxr1h* exons15-16 were assessed as CUGBP1 specific targets. Both *Cugbp1*$^{+/+}$/5-313 and *Cugbp1*$^{-/-}$/5-313 mice were tested in uninduced (-dox) and induced (+dox) conditions (n=5 per group). The percentage of exon inclusion is graphically represented. **(C)** Expression of embryonic myosin heavy chain (Myh3) was examined by qRT-PCR. Significant differences were $*P < 0.05$, $**P < 0.01$ and $***P < 0.005$ (t-test).

Figure 5. Improved Muscle Histopathology in CUGBP1$^{-/-}$/5-313 and correlation with levels of *Nkx2-5* mRNA. (A) Quadrceps femoris mucles were stained with hematoxylin and eosin. Both uninduced *Cugbp1*$^{+/+}$/5-313 and *Cugbp1*$^{-/-}$/5-313 mice have normal muscle histology. *Cugbp1*$^{-/-}$/5-313 (+dox) mice have milder histopathology compared to *Cugbp1*$^{+/+}$/5-313(+dox) mice. **(B)** Histopathology grading shows *Cugbp1*$^{-/-}$ has a beneficial effect (n=5); $**P < 0.002$ (Mann-Whitney U test). **(C)** *Nkx2-5* mRNA level was assessed by qRT-PCR and relative expression is presented. *Cugbp1*$^{-/-}$/5-313 mice (+dox) have five times less *Nkx2-5* mRNA than *Cugbp1*$^{+/+}$/5-313 mice (+dox) (n=5); $**P < 0.01$ (t-test).

Figure 6. MEF2A and C/EBPβ protein levels and expression of *MuRF1* mRNA are affected by absence of CUGBP1 in RNA toxicity. (A)Western blot for MEF2A. Numbers indicate relative

intensity of bands (n=4). Increased protein levels from *Cugbp1*$^{+/+}$/5-313 (+dox) are significantly reduced in *Cugbp1*$^{-/-}$/5-313 (+dox) mice ($P < 0.05$). **(B)** C/EBPβ LAP and **(C)** LIP isoforms were measured from western blots and relative protein expression are graphically represented. Both LAP and LIP levels were increased by RNA toxicity in induced 5-313 mice but not in *Cugbp1*$^{-/-}$/5-313 mice (n=5 per group). **(D)** Relative *MuRF1* mRNA expressions were measured by qRT-PCR. *Cugbp1*$^{+/+}$/5-313(+dox) mice (n=6) have five fold more *MuRF1* mRNA than uninduced mice (n=7) and these levels are reduced to 1.6 fold in *Cugbp1*$^{-/-}$/5-313(+dox) (n=5). $*P < 0.05$, $**P < 0.01$ and $***P < 0.005$ (t-test).